

Improved Oxygenation Dramatically Alters Metabolism and Gene Expression in Cultured Primary Mouse Hepatocytes

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Primary hepatocyte culture is an important *in vitro* system for the study of liver functions. *In vivo*, hepatocytes have high oxidative metabolism. However, oxygen supply by means of diffusion in *in vitro* static cultures is much less than that by blood circulation *in vivo*. Therefore, we investigated whether hypoxia contributes to dedifferentiation and deregulated metabolism in cultured hepatocytes. To this end, murine hepatocytes were cultured under static or shaken (60 revolutions per minute) conditions in a collagen sandwich. The effect of hypoxia on hepatocyte cultures was examined by metabolites in media and cells, hypoxia-inducible factors (HIF)-1/2 α western blotting, and real-time quantitative polymerase chain reaction for HIF target genes and key genes of glucose and lipid metabolism. Hepatocytes in shaken cultures showed lower glycolytic activity and triglyceride accumulation than static cultures, compatible with improved oxygen delivery and mitochondrial energy metabolism. Consistently, static cultures displayed significant HIF-2 α expression, which was undetectable in freshly isolated hepatocytes and shaken cultures. Transcript levels of HIF target genes (glyceraldehyde 3-phosphate dehydrogenase [*Gapdh*], glucose transporter 1 [*Glut1*], pyruvate dehydrogenase kinase 1 [*Pdk1*], and lactate dehydrogenase A [*Ldha*]) and key genes of lipid metabolism, such as carnitine palmitoyltransferase 1 (*Cpt1*), apolipoprotein B (*Apob*), and acetyl-coenzyme A carboxylase 1 (*Acc1*), were significantly lower in shaken compared to static cultures. Moreover, expression of hepatocyte nuclear factor 4 α (*Hnf4 α*) and farnesoid X receptor (*Fxr*) were better preserved in shaken cultures as a result of improved oxygen delivery. We further revealed that HIF-2 signaling was involved in hypoxia-induced down-regulation of *Fxr*. **Conclusion:** Primary murine hepatocytes in static culture suffer from hypoxia. Improving oxygenation by simple shaking prevents major changes in expression of metabolic enzymes and aberrant triglyceride accumulation; in addition, it better maintains the differentiation state of the cells. The shaken culture is, therefore, an advisable strategy for the use of primary hepatocytes as an *in vitro* model. (*Hepatology Communications* 2018;2:299-312)

Introduction

In vitro studies using isolated hepatocytes have greatly advanced our understanding of the (patho)-physiology of the liver. However, differentiated primary hepatocytes preserve only part of their properties when they are cultured *in vitro*. Currently available

immortalized hepatocyte and hepatoma cell lines cannot completely replace primary hepatocytes due to the limited degree of differentiation.

The maintenance of differentiated primary hepatocytes *in vitro* concerns specific aspects of cell isolation and culture procedure. Usually the isolation is performed by means of the two-step collagenase perfusion

Abbreviations: APOB, apolipoprotein B; ATP, adenosine triphosphate; cDNA, complementary DNA; CoA, coenzyme A; CPT1A, carnitine palmitoyltransferase 1A; DMOG, dimethylxalylglycine; EDTA, ethylene diamine tetraacetic acid; FSP27, fat-specific protein 27; FXR, farnesoid X receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT1, glucose transporter 1; HIF, hypoxia-inducible factor; HNF4 α , hepatocyte nuclear factor 4 α ; LDHA, lactate dehydrogenase A; MPA, metaphosphoric acid; mRNA, messenger RNA; NAD⁺, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; PBS, phosphate-buffered saline; PDK, pyruvate dehydrogenase kinase; PHD, prolyl hydroxylase domain; PPAR, peroxisome proliferator-activated receptor; PVDF, polyvinylidene difluoride; SREBP-1C, sterol regulatory element-binding transcription factor 1C; TBST, Tris-buffered saline with Tween 20; VHL, von Hippel-Lindau; vol, volume; wt, weight.

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technique.⁽¹⁾ The collagen sandwich culture technique is considered the optimal method for maintenance of differentiation on the basis of preserved active uptake and excretion mechanisms, formation of biliary networks, and polygonal morphology.⁽²⁻⁵⁾ Given the differentiated phenotypes, statically cultured primary hepatocytes often accumulate triglyceride spontaneously⁽⁶⁻⁹⁾; this signals a deranged metabolism and complicates their use in metabolic research. This metabolic derangement of static hepatocyte cultures warrants more attention as metabolic studies often do not compare the phenotype of cultured hepatocytes to freshly isolated cells or healthy liver.

Hepatocytes are equipped with abundant mitochondria and intrinsically have a high demand for oxygen.^(10,11) Isolated hepatocytes are usually cultured at a high density in hormone-supplemented medium to maintain cell–cell interactions and differentiated functions; however, it is often neglected that oxygen supply could be insufficient in hepatocyte cultures. In contrast to the *in vivo* situation where oxygen is delivered and buffered by circulating red blood cells from the portal vein and the hepatic artery, isolated hepatocytes in a standing culture dish rely on the slow diffusion of oxygen that must first cross the air–liquid interface. Therefore, cultured hepatocytes are prone to hypoxia and activation of hypoxia-inducible factor (HIF) signaling to launch adaptive responses. HIFs belong to the Per-Arnt-Sim family of helix–loop–helix transcription factors and are comprised of one α -subunit and one β -subunit. The regulation of active HIF levels relies on oxygen-sensitive α -subunits. During hypoxia,

the α -subunits translocate to the nucleus and heterodimerize with the constitutively expressed and stable β -subunit aryl hydrocarbon receptor nuclear translocator, forming the active transcription factor that binds hypoxia-responsive elements and increases transcription of the target genes.^(12,13) When oxygen is abundant, the α -subunit is prevented from translocating to the nucleus by proteasomal degradation. This phenomenon is the result of hydroxylation of α -subunits at specific proline residues by prolyl hydroxylase domain enzymes (PHD1, PHD2, PHD3) that use oxygen as a cosubstrate. The hydroxylated α -subunit is then polyubiquitinated by the von Hippel-Lindau (VHL) E3 ubiquitin ligase complex for subsequent degradation by the proteasomes.⁽¹⁴⁻¹⁶⁾ Among the three isoforms of α -subunits that have been described,⁽¹⁷⁾ HIF-1 α and HIF-2 α are best characterized. They share structural similarities and coordinate processes, such as erythropoiesis, angiogenesis, cell proliferation, inflammation, and energy metabolism.^(18,19) The metabolic hallmark of HIF activation is the switch from mitochondrial oxidative phosphorylation to glycolysis for adenosine triphosphate (ATP) production.⁽²⁰⁾

The importance of sufficient oxygen delivery in rat hepatocytes cultured in a bioreactor has been demonstrated in a study in which insufficient oxygenation significantly reduced the viability of hepatocytes and the synthesis of albumin and urea.⁽²¹⁾ Recently, Adam AAA, Van der Mark V, Donkers J, Wildenberg ME, Oude Elferink RP, Chamuleau RAFM, et al. (manuscript submitted) characterized a simple method of culturing human liver cell lines, relying on shaking. It

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was shown that a long-term shaken culture of liver cell line monolayers substantially increased their hepatic differentiation as well as their mitochondrial energy metabolism. In the present study, we investigate whether static cultures of primary mouse hepatocytes are in a hypoxic state and examine whether improving oxygen delivery to hepatocytes by shaking prevents hypoxic adaptive responses. Our results demonstrate that the shaken hepatocyte culture is a simple strategy to avoid activation of HIF signaling, aberrant glucose metabolism and triglyceride accumulation, and down-regulation of hepatocyte-enriched transcriptional factors in primary hepatocyte cultures.

Materials and Methods

Materials were purchased from Sigma-Aldrich unless otherwise indicated.

PRIMARY HEPATOCYTE ISOLATION

Primary mouse hepatocytes were isolated from wild-type C57BL/6 mice after overnight *ad libitum* feeding by a two-step collagenase perfusion method through the portal vein.⁽⁵⁾ The time from interruption of the blood circulation of the animal to the beginning of perfusion was always less than 1 minute to ensure optimal oxygenation. During the first step, the liver was perfused with Ca^{2+} -free Krebs buffer supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid–NaOH (pH 7.4) saturated with O_2/CO_2 (95:5 volume [vol]/vol) at 37 °C for 10 minutes. During the second step, the buffer was supplemented with collagenase type IV (0.25 mg/mL) and 1.7 mM CaCl_2 for an additional 10-minute perfusion. The softened liver was then transferred to a sterile plastic dish, and cells were dispersed with a coarse-toothed comb in cold Ca^{2+} -containing Krebs buffer and filtered through a 100- μm cell filter to remove cell clumps. The clump-free cell suspension was pelleted by centrifugation at 60g for 3 minutes at 4 °C and washed 4 times. For the last washing, the buffer was supplemented with 1% (weight [wt]/vol) bovine serum albumin. Viability was determined by the trypan blue exclusion test, and generally 30 million to 40 million cells were obtained with around 85% viability. A few aliquots of freshly isolated hepatocytes were immediately stored at –80 °C to serve as a reference for overnight hepatocyte cultures.

COLLAGEN-SANDWICH CULTURE

One million viable hepatocytes were plated in 2 mL of ice-cold Dulbecco's modified Eagle's media supplemented with 4.5 g/L glucose, 1 mM L-glutamine, 37.5 U/mL penicillin, 37.5 $\mu\text{g}/\text{mL}$ streptomycin, 1.75 g/L sodium bicarbonate, 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (pH 7.4), and 5% fetal bovine serum (Gibco) in 6-well plates (10^5 cells/ cm^2) precoated with 100 μL of 1.5 mg/mL (pH 7.4) type I rat-tail collagen (BD Biosciences). The plates were incubated for 3 hours in a static humidified incubator with 20% O_2 , 5% CO_2 at 37 °C to allow attachment of hepatocytes on the first collagen layer. To obtain a sandwich configuration,^(3,5) the medium was removed and the monolayer was washed once with prewarmed (37 °C) phosphate-buffered saline (PBS) and a second 100- μL collagen layer was immediately added to cover the hepatocytes. The plates were incubated for 3–5 minutes in the incubator to gel the second collagen layer, and 2 mL prewarmed (37 °C) medium containing 1% fetal bovine serum was carefully added to the sandwiched hepatocytes for overnight culture. The plates were allowed to rest in the incubator for 15 minutes before being randomly assigned to static cultures (20% O_2) or to shaken cultures (5% or 20% O_2) on an orbital shaker at a rate of 60 revolutions per minute (rpm) in a 5% CO_2 incubator. As a positive control for HIF signaling, 1 mM dimethylxalylglycine (DMOG) was added to the shaken culture under 20% O_2 .

COLLECTION OF MEDIUM AND CELL SAMPLES FOR METABOLITE MEASUREMENTS

For measurement of glucose, pyruvate, and L-lactate, 50 μL of spent medium from overnight hepatocyte cultures was harvested into Eppendorf tubes containing 75 μL ice-cold 5% (wt/vol) metaphosphoric acid (MPA).⁽²²⁾ The mixture was centrifuged at 14,000g for 10 minutes at 4 °C, and the supernatant was stored without neutralization at 4 °C. For measurement of triglyceride and glycogen, the residual medium was removed and the cells were harvested in 100 μL ice-cold 2% Triton X-100. The lysate was centrifuged at 14,000g for 10 minutes, and the supernatant was stored at –20 °C until analysis. For enzymatic determination of glucose, pyruvate, and L-lactate, the reaction buffers were designed as such

that the optimal assay pH was obtained with the addition of the indicated volume of the acidic deproteinized samples.

Glucose Determination

Medium glucose after overnight culture was assayed enzymatically according to Blake and McLean⁽²³⁾ with modification for the microplate reader. Briefly, 10 μ L of the MPA-deproteinized sample was mixed with 150 μ L assay buffer (166.67 mM Na₂HPO₄-NaH₂PO₄ buffer pH 6.0, 2.33 mM 4-aminoantipyrine, 2.33 mM Na *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine, and 0.67 U/mL horseradish peroxidase [Roche]). After determining the background absorbance at 555 nm, 50 μ L of 8 U/mL glucose oxidase (*Aspergillus niger*, type II-S; prepared in 100 mM Na₂HPO₄-NaH₂PO₄ buffer pH 6.0) was added and absorbance was followed every 2 minutes until the reaction was completed (within 1 hour). Standard glucose solutions (0.3125 mM to 4 mM) were prepared in 3% MPA and assayed together with samples. Samples were diluted in 3% MPA if necessary.

Pyruvate Determination

Medium pyruvate was assayed enzymatically according to Zhu et al.⁽²⁴⁾ with modifications. The assay was performed at 37°C in the CLARIOstar microplate reader (BMG LABTECH). Briefly, 20 μ L of the MPA-deproteinized sample was mixed with 150 μ L assay buffer (200 mM K₂HPO₄-KH₂PO₄ buffer pH 8.0, 1.33 mM MgCl₂, 1.33 mM ethylene diamine tetraacetic acid [EDTA]-NaOH pH 8.0, and 0.5 mM homovanillic acid). After determining the background fluorescence at $\lambda_{\text{excitation [ex]}}/\lambda_{\text{emission [em]}} = 320/450$ nm, 50 μ L start solution (2 U/mL pyruvate oxidase and 5 U/mL horseradish peroxidase) was added and fluorescence was followed every 2 minutes until the reaction was completed (within 1 hour). Standard pyruvate solutions (3.9 μ M to 500 μ M) were prepared in 3% MPA and assayed together with samples.

L-Lactate Determination

Medium L-lactate was measured enzymatically according to Marbach and Weil⁽²²⁾ with modification for the microplate reader. The assay was performed at 37°C in the CLARIOstar microplate reader (BMG LABTECH). Briefly, 5 μ L of MPA-deproteinized sample was mixed with 130 μ L 0.5 M glycine-0.4 M hydrazine buffer (pH 9.0) and 20 μ L 2.7 mM oxidized

nicotinamide adenine dinucleotide (NAD⁺). After determining the background fluorescence at $\lambda_{\text{ex}}/\lambda_{\text{em}} = 340/450$ nm, 50 μ L 27.5 U/mL L-lactate dehydrogenase from rabbit muscle (Roche) in 0.5 M glycine-0.4 M hydrazine buffer (pH 9.0) was added and fluorescence was followed every 2 minutes until the reaction was completed (within 1 hour). Standard L-lactate solutions (7.8 μ M to 1,000 μ M) were prepared in 3% MPA and assayed together with samples. Samples were diluted in 3% MPA as necessary.

Triglyceride and Glycogen Determination

Triglyceride was determined by a two-step procedure using a commercially available Trig/GB-kit (Roche, the Netherlands). Briefly, 20 μ L Triton X-100 lysate was mixed with 100 μ L glycerol-blanking solution (150 mM Tris-HCl pH 7.6, 17.5 mM MgSO₄, 10 mM EDTA, 3.5 mM chlorophenol, 6 μ M K₃Fe(CN)₆, 0.15% sodium cholate, 0.12% hydroxypolyethoxy-n-alkanes, 1 mM ATP, 0.4 U/mL glycerol kinase, 5 U/mL glycerol phosphate oxidase, 0.3 U/mL horseradish peroxidase) for 15 minutes at 37°C. After determining the background absorbance at 490 nm, 100 μ L lipase-chromophore solution (150 mM Tris-HCl pH 7.6, 17.5 mM MgSO₄, 10 mM EDTA, 3.5 mM chlorophenol, 6 μ M K₃Fe(CN)₆, 0.15% sodium cholate, 0.12% hydroxypolyethoxy-n-alkanes, 1 mM ATP, 6 U/mL lipase, 0.7 mM 4-aminophenazone) was added. Absorbance at 490 nm was then followed every 2 minutes until the reaction was completed. For glycogen determination, 50 μ L Triton X-100 lysate was mixed with an equal volume of 0.2 N NaOH and incubated in an 80°C water bath with shaking for 30 minutes to degrade monosaccharides and disaccharides. Next, the alkaline lysate was cooled and 10 μ L of 2 M acetic acid was added to the samples to bring the pH to 4.75. Subsequently, 10 μ L of 10 U/mL amyloglucosidase from *Aspergillus niger* (prepared in 50 mM sodium acetate buffer [pH 4.75]) was added, and the mixture was incubated at 60°C for 45 minutes to degrade glycogen to glucose.⁽²⁵⁾ The reaction was stopped and deproteinized by adding 180 μ L ice-cold 5% MPA. The mixture was then centrifuged at 14,000g for 10 minutes, and the supernatant was assayed for glucose, as described above. Protein concentration in the samples and blank wells with only a collagen sandwich were measured by the bicinchoninic acid assay.⁽²⁶⁾ The triglyceride and glycogen measurements were normalized to protein content.

SODIUM DODECYL SULFATE–POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN BLOT

Overnight cultures of primary murine hepatocytes were harvested directly in nuclear extraction buffer (420 mM NaCl, 20% [wt/vol] glycerol, 5 mM MgCl₂, 5 mM ethylene glycol tetraacetic acid, 0.5% NP-40, 20 mM Tris-HCl pH 8.0) freshly supplemented with complete EDTA-free protease inhibitor cocktail (Roche), PhosSTOP phosphatase inhibitor cocktail (Roche), and 1 mM dithiothreitol. Frozen pellets of freshly isolated hepatocytes were lysed directly in nuclear extraction buffer. The samples were incubated on a rotor for 30 minutes at 4 °C and then centrifuged at 14,000g for 10 minutes at 4 °C. The supernatant was harvested for sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Protein concentration was determined by the bicinchoninic acid assay. Forty micrograms of protein were electrophoresed on an 8% sodium dodecyl sulfate–polyacrylamide gel, transferred to a polyvinylidene difluoride (PVDF) membrane by semidry blotting, and blocked overnight in 5% nonfat milk/PBS with 0.05% (wt/vol) Tween 20. For immunodetection, the PVDF membrane was incubated with primary antibody for 1 hour at room temperature (see [Supporting Table S1](#) for the list of antibodies and dilutions used). The PVDF membrane was washed 3 times with Tris-buffered saline with 0.05% (wt/vol) Tween 20 (TBST), incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse immunoglobulin G antibody (Bio-Rad Laboratories) for 1 hour, and washed 3 times with TBST. All antibodies were diluted in 1% nonfat milk/TBST, and incubation was performed at room temperature. The PVDF membrane was developed with homemade enhanced chemiluminescence reagents (100 mM Tris-HCl pH 8.5, 1.25 mM luminol, 0.2 mM p-coumarin, and freshly added 3 mM H₂O₂) and detected by Image-Quant LAS 4000 (GE Healthcare Life Sciences).

RNA ISOLATION, COMPLEMENTARY DNA SYNTHESIS, AND QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

Total RNA was isolated from freshly isolated or overnight-cultured hepatocytes with TRI Reagent (Sigma). RNA concentration was measured by

spectrophotometry at 260 nm using a Nanodrop 1000 (Thermo Scientific). We treated 2 µg total RNA with deoxyribonuclease I (Promega) and reversely transcribed into complementary DNA (cDNA) with deoxythymidine oligomer, random hexamers, and RevertAid reverse transcriptase (Thermo Scientific) in a reaction volume of 20 µL. The resulting cDNA was 10-fold diluted with water, and 4 µL of the diluted cDNA was used as a template for quantitative reverse-transcription polymerase chain reaction (PCR) by a Roche Lightcycler 480 II instrument using the SensiFAST SYBR No-ROX kit (Bioline). Initial fluorescent values were calculated by LinRegPCR³ (version 2013.0; Academic Medical Center, Amsterdam). Expression levels were normalized to 18S ribosomal RNA. Please refer to [Supporting Table S2](#) for primer sequences used in the quantitative reverse-transcription PCR.

OIL RED O STAINING

Primary mouse hepatocytes were cultured in a collagen sandwich in 12-well plates either statically or with shaking for 24 and 48 hours. Cells were washed twice with ice-cold PBS and fixed with 4% paraformaldehyde/5% sucrose/50 mM K₂HPO₄-KH₂PO₄ buffer (pH 7.4) for 1 hour at room temperature. The fixed cells were then washed 2 times with 1 mL PBS and 2 times with 1 mL deionized water. To each well, 400 µL working solution of Oil Red O (0.3% Oil Red O in 36% triethyl phosphate) was added. After incubating at room temperature for 30 minutes, cells were washed 3 times with deionized water. Bright-field images were taken by a DMi8 microscope (Leica).

STATISTICS

All results are given as mean ± SD. Statistical significance was determined by a two-tailed Student *t* test or one-way analysis of variance followed by Tukey's test using GraphPad Prism 5 (GraphPad Software, LaJolla, CA) with an α error of 0.05.

Results

LIPID AND CARBOHYDRATE METABOLISM IN SHAKEN VERSUS STATIC HEPATOCYTE CULTURES

In order to investigate whether oxygen delivery is important for *in vitro* hepatocyte cultures, primary

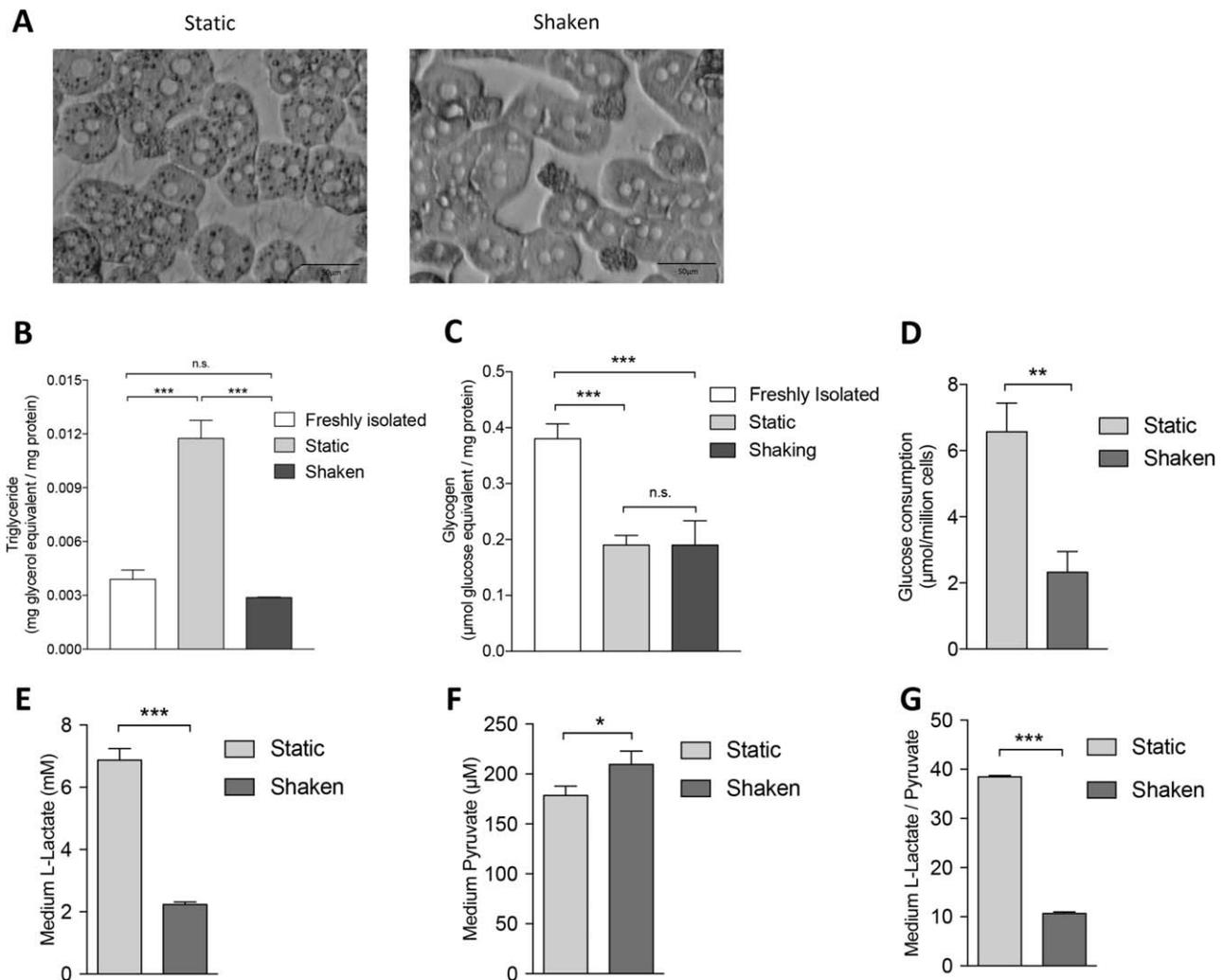


FIG. 1. Static culture promotes lipid accumulation and glycolysis in primary mouse hepatocytes. (A) Representative light microscopy images of hepatocytes in static or shaken culture 20 hours after attachment. (B) Triglyceride or (C) glycogen of freshly isolated hepatocytes and hepatocytes after overnight in static and shaken culture were assayed. Overnight medium samples of primary mouse hepatocytes in static and shaken culture were collected for enzymatic determination of (D) glucose, (E) L-lactate, and (F) pyruvate. (G) The lactate and pyruvate results were used to calculate the lactate-to-pyruvate ratio. Results are representative of three independent experiments of triplicate determinations. The data (B-G) represent mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Abbreviation: n.s., not significant.

mouse hepatocytes were sandwiched in two collagen layers and cultured statically (static cultures) or with continuous shaking at 60 rpm (shaken cultures) under 20% O_2 . Freshly isolated hepatocytes (during isolation perfused with medium equilibrated with 95% O_2 and 5% CO_2) served as reference. Because insufficient oxygen delivery could limit lipid oxidation and promote glycolysis, we studied the metabolism of lipid and glucose in both static and shaken cultures. Light microscopy revealed lipid droplet-like structures in the static cultures but not in the shaken culture (Fig. 1A).

To verify, we determined the triglyceride content biochemically and found that the triglyceride content of the static culture was 4-fold higher than that of the shaken culture (Fig. 1B). Of note, the triglyceride content of the shaken culture was not different from that of the freshly isolated hepatocytes. In contrast, the amount of glycogen was similar between static and shaken cultures and was about 50% that of the freshly isolated hepatocytes from mice fed *ad libitum* (Fig. 1C).

Overnight media samples from both static and shaken cultures were collected for determination of

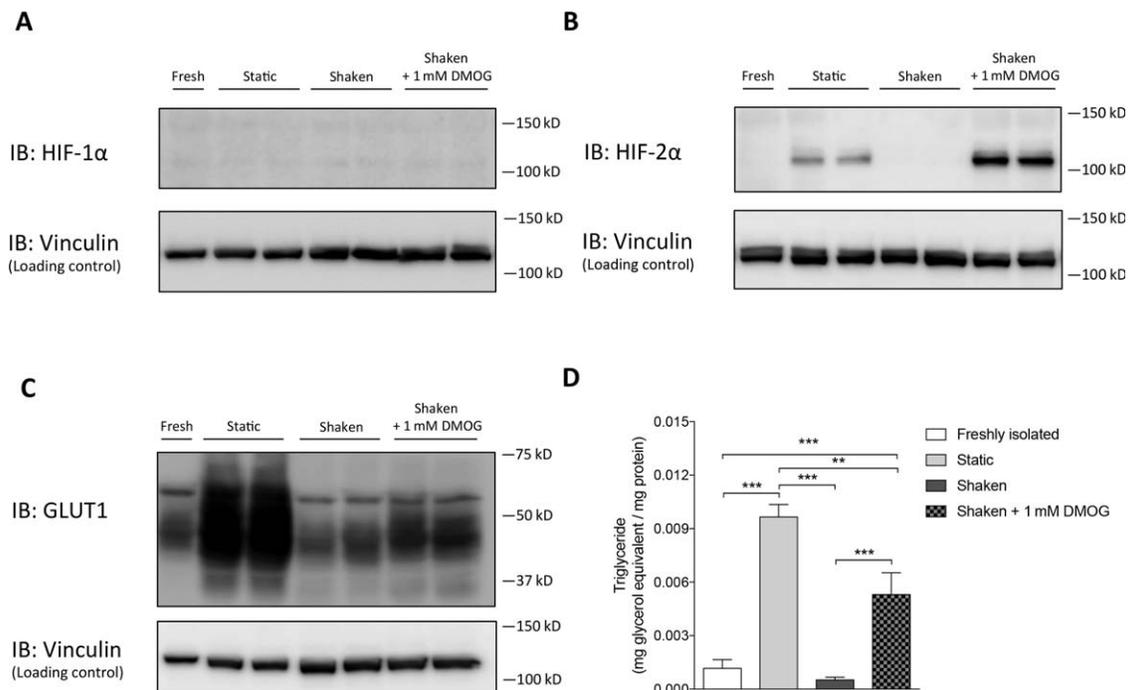


FIG. 2. Activation of HIF signaling causes lipid accumulation in statically cultured primary mouse hepatocytes. Protein lysates of primary mouse hepatocytes after overnight static and shaken culture were subjected to immunoblot for (A) HIF-1 α , (B) HIF-2 α , and (C) the HIF-target GLUT1. (D) Lipid from overnight static and shaken hepatocyte cultures in triplicate was extracted and enzymatically determined. Results are representative of three independent experiments. The data in D represent mean \pm SD. ** $P < 0.01$, *** $P < 0.001$. Abbreviation: IB, immunoblot.

glucose, lactate, and pyruvate. Compared with shaken cultures, glucose uptake of the static culture was 2.8-fold higher than that of the shaken culture (Fig. 1D), medium lactate concentration was 3.1-fold higher (Fig. 1E), and medium pyruvate concentration was 85% that of the shaken culture (Fig. 1F). Consequently, the lactate-to-pyruvate ratio in the medium (Fig. 1G) was significantly (3.6-fold) higher in static cultures, indicating a higher reduced NAD (NADH)/NAD⁺ ratio in the cytosol of the cells. To examine whether the improved metabolism was the result of the shaking procedure or the result of improved oxygen delivery, hepatocytes were cultured with shaking under hypoxic conditions (5% O₂). Consistently, shaken hepatocytes cultured under 5% O₂ had increased lactate secretion, reduced pyruvate secretion, and an increased lactate-to-pyruvate ratio in the medium, resembling that of the static hepatocyte culture under 20% O₂ (Supporting Fig. S1). These data demonstrated that, as a result of improved oxygen delivery, shaken hepatocyte cultures had a higher capacity in oxidizing fatty acid and pyruvate than the statically cultured hepatocytes.

HYPOXIA CAUSES ACTIVATION OF HIF-2 SIGNALING AND LIPID ACCUMULATION IN STATICALLY CULTURED PRIMARY MOUSE HEPATOCYTES

To verify that the metabolic differences between static and shaken hepatocyte cultures were caused by hypoxia, we examined whether HIF signaling was activated in static hepatocyte cultures compared to shaken hepatocyte cultures. As a control, shaken cells were also cultured in the presence of 1 mM DMOG, a cell-permeant 2-oxoglutarate analogue that inhibits 2-oxoglutarate-dependent dioxygenases (including prolyl hydroxylase domain enzymes), to activate HIF signaling under normoxia.⁽¹⁴⁾ Treatment with 1 mM DMOG effectively increased expression of HIF-target genes (Supporting Fig. S2), demonstrating the presence of HIF signaling in primary mouse hepatocytes. Immunoblotting for HIF-1 α failed to reveal any signal, even under DMOG treatment (Fig. 2A). In contrast,

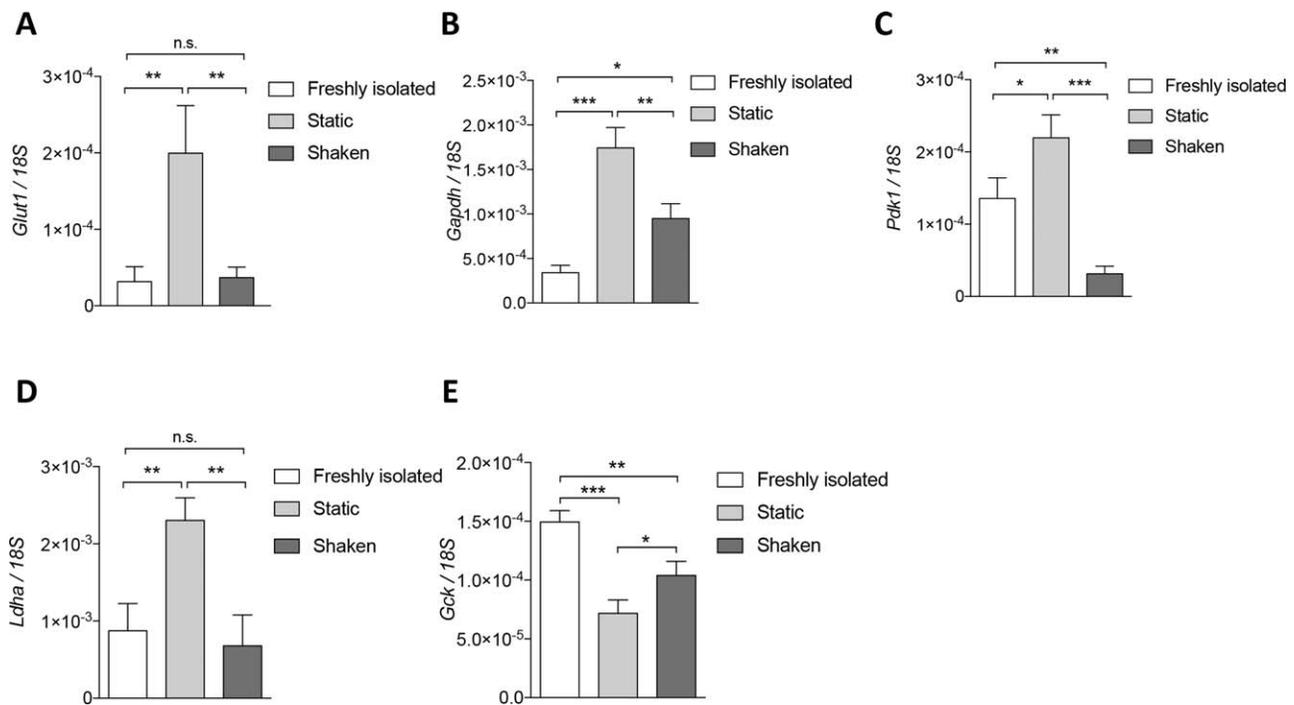


FIG. 3. Shaken cultures reverted increased expression of glycolysis-related genes in static cultures. Transcript levels of HIF-responsive genes and glycolysis-related genes were examined in freshly isolated primary mouse hepatocytes and overnight static and shaken cultures by quantitative reverse-transcription polymerase chain reaction. (A) *Glut1*, (B) *Gapdh*, (C) *Pdk1*, (D), *Ldha*, (E) *Gck*. Results are representative of three independent experiments of triplicate determinations. The data represent mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Abbreviation: n.s., not significant.

HIF-2 α was found to be up-regulated in both the static culture and the DMOG-treated shaken culture while it was not detected in freshly isolated hepatocytes or the shaken culture (Fig. 2B). Consistently, the protein level of glucose transporter 1 (GLUT1), an HIF signaling target gene, was substantially higher in the static cultures and DMOG-treated shaken cultures (Fig. 2C). Activating HIF signaling with DMOG not only increased the GLUT1 level but also induced substantial accumulation of triglyceride in the shaken culture (Fig. 2D). Interestingly, while the DMOG-treated shaken culture (i.e., activation of HIF signaling under normoxia) had a higher level of HIF-2 α than the static culture, the latter had a higher expression of GLUT1 and accumulated more triglycerides. This suggested that the metabolic derangements in the static culture were mediated by both HIF-dependent and HIF-independent mechanisms (e.g., hypoxia *per se*). While it is preferred that the isolated hepatocytes are used for metabolic studies as soon as possible, we found significantly lower protein levels of both HIF-2 α (Supporting Fig. S3A) and GLUT1 (Supporting Fig. S3B) in the shaken hepatocyte culture compared

to the static hepatocyte culture up to 48 hours in culture, suggesting that the shaking procedure could be applied in long-term models to improve oxygenation. Consistently, Oil Red O staining revealed significantly less lipid accumulation in the shaken culture than in the static culture (Supporting Fig. S3C).

EXPRESSION OF GLYCOLYSIS-RELATED GENES IN STATIC AND SHAKEN CULTURES

To further investigate the effect of static culturing on glycolysis, we examined the expression of a panel of glycolysis-related genes. Transcript levels of *Glut1*, glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), pyruvate dehydrogenase kinase 1 (*Pdk1*), and lactate dehydrogenase A (*Ldha*) in static cultures were 5.4-fold, 1.8-fold, 7-fold, and 3.4-fold higher than in shaken cultures, respectively (Fig. 3A-D). Up-regulation of these four well-established HIF-responsive genes was consistent with the activated HIF signaling in statically cultured hepatocytes. The transcript

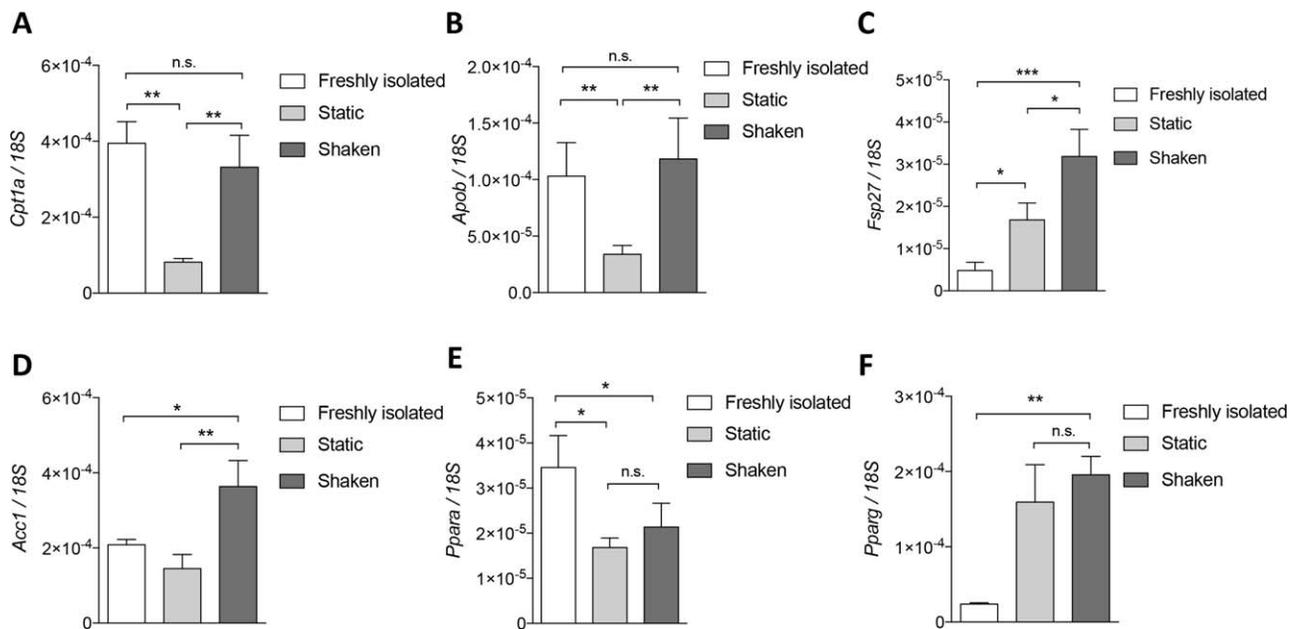


FIG. 4. Effect of the shaken culture on expression of genes related to lipid metabolism. Transcript levels of genes related to lipid metabolism were examined in freshly isolated primary mouse hepatocytes and overnight static and shaken cultures by quantitative reverse-transcription polymerase chain reaction. (A) *Cpt1a*, (B) *Apob*, (C) *Fsp27*, (D), *Acc1*, (E) *Ppara*, (F) *Pparg*. Results are representative of three independent experiments of triplicate determinations. The data represent mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Abbreviation: n.s., not significant.

of glucokinase (*Gck*), a liver-specific hexokinase isozyme, was reduced in both static and shaken cultures to 48% and 70% that of the freshly isolated hepatocytes, respectively (Fig. 3E). Importantly, the hypoxic shaken culture (5% O₂) also had increased *Glut1* expression to a similar extent as the static culture under 20% O₂, suggesting that the differences in gene expressions between the static and shaken cultures were not caused by the shaking procedure but by differences in oxygen delivery (Supporting Fig. S4A).

EXPRESSION OF GENES INVOLVED IN LIPID METABOLISM

To investigate whether altered expression of enzymes of lipid metabolism contributed to the difference in the triglyceride content between static and shaken hepatocyte cultures, transcript levels of genes regulating the synthesis, storage, oxidation, and export of lipids were measured. The most evident difference in gene expression was observed for carnitine palmitoyltransferase 1A (*Cpt1a*) (Fig. 4A), which is localized at the outer mitochondrial membrane and is the first and rate-limiting enzyme for carnitine-dependent

transport of long chain fatty acids into the mitochondria for β -oxidation.⁽²⁷⁾ The shaken cultures presented similar transcript levels of *Cpt1a* compared to the freshly isolated hepatocytes (both about 4-fold higher than that of the static cultures). The *Cpt1a* expression in the hypoxic shaken culture (5% O₂) was similar to the static culture and lower than the normoxic shaken culture, confirming the importance of oxygen supply (Supporting Fig. S4B). Similarly, apolipoprotein B (ApoB)-100 expression in shaken cultures was 3-fold higher than in static cultures, and no differences were found when compared to freshly isolated hepatocytes (Fig. 4B). Fat-specific protein 27 (*Fsp27*), a lipid-droplet-associated protein that is thought to promote lipid accumulation,⁽²⁸⁾ was 4-fold up-regulated in the static cultures when compared with freshly isolated hepatocytes (Fig. 4C). Unexpectedly, the messenger RNA (mRNA) level of *Fsp27* was 2-fold higher in shaken cultures than in hepatocytes in static culture and 7-fold higher than in freshly isolated hepatocytes. The transcript of acetyl-CoA carboxylase 1 (*Acc1*), a key enzyme for lipid synthesis, was 1.5-fold higher in shaken cultures compared to freshly isolated or statically cultured hepatocytes (Fig. 4D). Peroxisome proliferator-activated receptor α (*Ppara*), known to

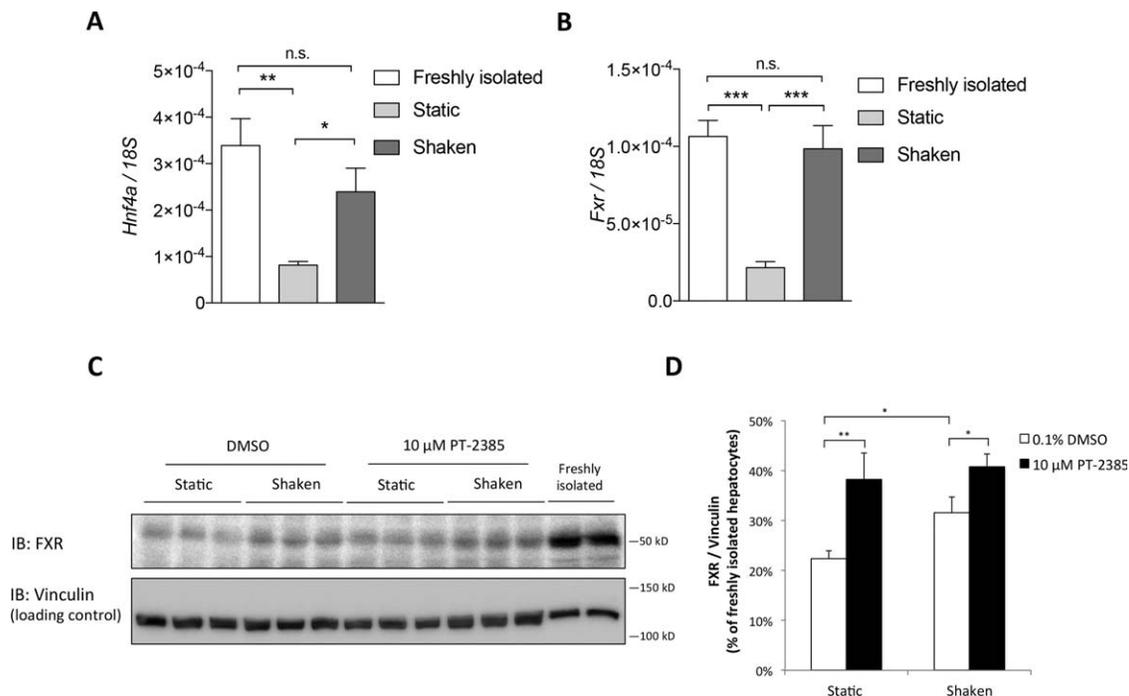


FIG. 5. Expression of *Hnf4α* and *Fxr* in static and shaken cultures of primary mouse hepatocytes. Transcript levels of (A) *Hnf4α* and (B) *Fxr* were examined in freshly isolated primary mouse hepatocytes and overnight static and shaken cultures by quantitative reverse-transcription polymerase chain reaction. (C) Immunoblot for FXR in freshly isolated primary mouse hepatocyte and overnight static and shaken cultures. (D) Immunoblot was quantified by ImageJ. Results are representative of at least two independent experiments of triplicate determinations. The data (A, B, and D) represent mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Abbreviations: DMSO, dimethyl sulfoxide; IB, immunoblot; n.s., not significant.

promote uptake, utilization, and oxidation of fatty acids, was 2-fold lower in hepatocyte cultures compared to freshly isolated cells. No significant differences were found between shaken and static cultures (Fig. 4E). Reciprocal results were found for peroxisome proliferator-activated receptor γ (*Pparg*), a transcriptional regulator for lipid storage (Fig. 4F). The expression of lipid synthesis regulator sterol regulatory element-binding transcription factor 1C (*Srebp-1c*) was not affected in cultured hepatocytes compared to freshly isolated ones and was not affected by shaking the plates during culture (data not shown).

EXPRESSIONS OF HEPATOCYTE NUCLEAR FACTOR 4 α AND FARNESOID X RECEPTOR WERE SUPPRESSED UNDER HYPOXIA

Lastly, we examined whether oxygen delivery affects the expression of two hepatocyte-enriched nuclear transcription factors: hepatocyte nuclear factor 4 α

(HNF4 α) and farnesoid X receptor (FXR). HNF4 α orchestrates hepatocyte differentiation and metabolism of carbohydrate and lipid, while FXR regulates the metabolism of bile salts and cholesterol. Compared to static cultures, the transcript level of *Hnf4α* was 3-fold higher in shaken cultures (Fig. 5A) and the transcript level of *Fxr* was 4-fold higher (Fig. 5B). Importantly, the transcript levels of both *Hnf4α* and *Fxr* in the shaken cultures and freshly isolated hepatocytes were similar. In addition, the expression of *Hnf4α* and *Fxr* in the hypoxic shaken culture (5% O₂) was similar to the static culture and lower than the normoxic shaken culture, confirming the importance of oxygen supply (Supporting Fig. S4C,D). Because HIF-2 α was strongly induced in static cultures, we examined if selectively inhibiting dimerization of HIF-2 α and HIF-1 β (an aryl hydrocarbon receptor nuclear translocator) by PT-2385⁽²⁹⁾ could increase FXR protein expression in the static cultures. Similar to *Fxr* transcript levels, the shaken cultures had higher FXR protein than the static cultures. Importantly, treatment with PT-2385 increased FXR protein in both static

and shaken cultures to about 40% of the freshly isolated hepatocytes (Fig. 5C,D), indicating that HIF-2 signaling underlies hypoxia-induced down-regulation of FXR, but FXR is also regulated by other hypoxia-independent mechanisms. Overall, these results demonstrated that the shaking procedure, by improving oxygen delivery, improved the expressions of *Fxr* and *Hnf4 α* .

Discussion

In this study we showed that the conventional static culture of primary mouse hepatocytes has limited capacity in oxygen delivery; this subjects the cells to hypoxia, activation of HIF signaling, enhanced glycolysis, and excessive triglyceride accumulation. These phenomena complicate, if not exclude, the use of conventionally cultured primary mouse hepatocyte for metabolic studies. We demonstrated that the shaken cultures represent a simple and effective solution to reverse the adverse phenotypes associated with static cultures.

The availability of oxygen is of decisive importance for hepatocytes due to their high intrinsic metabolic activity. We demonstrated in the present study that statically cultured hepatocytes present both metabolic and transcriptional signatures of hypoxia. First, the strong induction of HIF-2 α protein statically cultured hepatocytes indicates the presence of hypoxia (Fig. 2B). Although we could not detect HIF-1 α in either the static culture or the DMOG-treated shaken culture (Fig. 2A), we could not completely rule out the contribution of HIF-1 α to the observed differences in metabolism and gene expression. HIF-1 α and HIF-2 α have an overlapping but not identical spectrum of target genes.⁽³⁰⁾ Second, HIF target genes, such as GLUT1, GAPDH, LDHA, and PDK1, were up-regulated in static cultures (Fig. 4A-D), confirming active HIF signaling. Up-regulation of PDK1 suppresses activity of the pyruvate dehydrogenase complex by phosphorylating pyruvate dehydrogenase,^(20,31) thereby reducing the conversion of pyruvate to acetyl-CoA during hypoxia. Together with PDK1, the up-regulation of GAPDH, LDHA, and GLUT1 are important adaptive hypoxic responses that ensure increased capacity for glycolysis and reduced flux to Krebs cycle and oxidative phosphorylation. Consistently, there was a 2-fold increase in glycolytic flux (Fig. 1D,E). Third, the lactate-to-pyruvate ratio in the medium was 3-fold higher (Fig. 1G), indicating an

increased cytosolic NADH/NAD⁺ ratio and diminished NADH shuttling into mitochondria as a result of the limited availability of oxygen. In contrast to the static cultures, HIF-2 α in the shaken cultures was undetectable, and the expression of HIF target genes was comparable with that of the freshly isolated hepatocytes. Importantly, the improved metabolic phenotypes and the prevention of HIF activation in the shaken cultures were not caused by the shaking procedure itself because these beneficial effects were absent when the shaken cultures were subjected to hypoxic ambience (5% O₂) (Supporting Figs. S1 and S4). These observations show that, while both static and shaken culture conditions had the same amount of atmospheric oxygen available, shaking the plates prevents hypoxia by improving the delivery of oxygen to the hepatocytes.

In addition to activation of HIF signaling and enhanced glycolysis, we observed that statically cultured hepatocytes accumulate an excessive amount of triglyceride. Lipid accumulation in the static hepatocyte culture involves both altered metabolite flux and transcriptional regulation.^(32,33) During hypoxia, oxidative phosphorylation comes to a halt, causing incomplete oxidation of fatty acids by β -oxidation and accumulation of acetyl-CoA and Krebs cycle intermediates in mitochondria. Excessive mitochondrial acetyl-CoA is shuttled (in the form of citrate) to cytosol and serves as starting material for *de novo* lipogenesis. Reduced ATP production from oxidative phosphorylation leads to a compensatory increase of glycolytic flux to maintain cellular ATP through the allosteric regulation of glycolytic enzymes. Up-regulation of glycolysis and increased glucose uptake provide the lipid backbone glycerol-3-phosphate and facilitate generation of nicotinamide adenine dinucleotide phosphate by the pentose phosphate pathway.⁽³⁴⁾ In the static hepatocyte culture, the hypoxia-induced up-regulation of *Glut1* (Fig. 3A) is likely to facilitate *de novo* lipogenesis. Of note, while HIF signaling increases maximal capacity of glycolysis and supports lipid synthesis, it is hypoxia *per se* not HIF signaling that drives the observed changes in the metabolism of glucose and lipid in statically cultured primary mouse hepatocytes. Consistently, we found no effect of the HIF-2 inhibitor PT-2385, which does not resolve hypoxia, on the metabolism of lipid and glucose of the statically cultured hepatocytes (data not shown).

A survey of genes involved in lipid metabolism revealed a complex picture. *Cpt1a*, a regulatory step in fatty acid transport into mitochondria, is strongly

down-regulated in static hepatocyte cultures, while shaking effectively restores the expression level to that of freshly isolated hepatocytes (Fig. 4A). This is similar to the work of Rankin et al.,⁽³⁵⁾ where *Cpt1a* was up-regulated in *Vhl*^{-/-} liver in an HIF-2 α -dependent fashion. Similarly, *ApoB* is also down-regulated under hypoxia (Fig. 4B). Unlike in human hepatocytes, the *ApoB* mRNA in rodent hepatocytes is subjected to cystine-to-uridine base editing by ApoB editing enzyme catalytic subunit 1. ApoB-100, translated from the unedited mRNA, is indispensable in initiation of very low-density lipoprotein biogenesis.^(36,37) Therefore, decreased *ApoB* can contribute to lipid accumulation by decreased biogenesis and secretion of very low-density lipoprotein. Surprisingly, *Acc1*, which catalyzes the committed step for fatty acid synthesis, is lower in the static culture in comparison to the shaken culture (Fig. 4D). Our data are in line with what was found by Nishiyama et al.⁽³⁸⁾ in mouse liver during ethanol-induced hypoxia and with the work of Rankin et al.⁽³⁵⁾ in which transcript levels of lipogenic enzymes were reduced by HIF-2 signaling in *Vhl*^{-/-} mouse livers. The expression of these lipogenic enzymes is likely altered by mechanisms other than transcriptional regulation of SREBP-1c as we did not observe differential expression of Srebp-1c between static and shaken cultures (data not shown). We also examined *Fsp27*, a PPAR-targeted lipid droplet protein that facilitates lipid storage. *Fsp27*^{-/-} mice have strongly reduced hepatic triglycerides, whereas overexpression of this gene induces hepatic steatosis.^(39,40) Unexpectedly, statically cultured hepatocytes have a lower expression of *Fsp27* than hepatocytes in the shaken culture (Fig. 4C). Apparently in cultured hepatocytes, FSP27 is not the rate-controlling factor in triglyceride accumulation.

Although no gene involving lipogenesis and lipid oxidation has been identified as direct target of HIF signaling,⁽⁴¹⁾ there is accumulating evidence indicating that sustained HIF signaling leads to hepatic steatosis underlying several metabolic liver diseases, including nonalcoholic fatty liver disease and alcoholic liver disease.⁽¹⁹⁾ In a mouse model overexpressing HIF-1 α and HIF-2 α variants, which cannot be prolyl hydroxylated (and therefore are constitutively active), hepatic steatosis is observed.⁽⁴²⁾ Rankin et al.⁽³⁵⁾ showed that *Vhl*^{-/-} mice develop hepatic steatosis, reduced expression of genes involved in β -oxidation (acyl-CoA synthetase long-chain family member 1 [*Acs1l*] and *Cpt1a*), and increased expression of adipose differentiation-related protein (*Adfp*), a lipid droplet-binding protein in hepatocytes. The authors further demonstrated that

deletion of HIF-2 α but not HIF-1 α effectively reversed hepatic steatosis in *Vhl*^{-/-} mice, suggesting that HIF-2 α is the main player in hypoxic steatosis. Using a temporal tamoxifen-inducible *Vhl*^{f/f-Alb-CreERT2} mouse model, Qu et al.⁽⁴³⁾ showed that liver-specific disruption of VHL led to sustained hepatic steatosis, liver inflammation, and fibrosis. Importantly, the accumulation of lipid can be observed within 24 hours after disruption of VHL. These observations are consistent with our finding and time frame that HIF-2 α is strongly induced in statically cultured hepatocytes and that lipid accumulates in static hepatocyte cultures after overnight culture. Importantly, treatment of the shaken cultures with DMOG also induced HIF-2 α and resulted in substantial accumulation of lipid (Fig. 2).

The dedifferentiation in primary hepatocyte culture, characterized by progressive loss of *in vivo* phenotypes and functions, is a constant challenge for *in vitro* studies using primary hepatocytes.⁽⁴⁴⁾ The deterioration of specialized functions usually results from altered transcriptional regulation induced by isolation *per se* and subsequent culturing. For example, HNF4 α is usually down-regulated in cultured hepatocytes,⁽⁴⁵⁾ and FXR has been shown to be greatly reduced under hypoxia in the human hepatoma cell line HepG2.⁽⁴⁶⁾ One major finding of the current work is that shaking improves the expression of the transcription regulators *Hnf4 α* and *Fxr* in cultured hepatocytes (Fig. 5A,B; Supporting Fig. S4C,D). HNF4 α plays an important role in biotransformation and in the metabolism of glucose and lipid.^(47,48) The maintenance of HNF4 α in *in vitro* culture is therefore desirable for drug toxicity and metabolic studies. FXR plays important roles in metabolism of glucose, lipid, cholesterol, and bile acids and represents a potential target to treat hepatic steatosis.^(49,50) Consequently, FXR stabilization in the shaken culture would improve bile salt signaling studies in primary hepatocytes. Interestingly, the HIF-2 α -specific inhibitor PT-2385 increases FXR expression in static cultures but does not restore it to the level of freshly isolated hepatocytes. Taken together, while hypoxia played an important role in down-regulating *Fxr* and *Hnf4 α* in cultured hepatocytes, additional oxygen-independent regulatory mechanisms were likely present.

Many of the genes analyzed in this study are strongly regulated by hormones, like insulin, glucagon, cortisol, and epinephrine, which are often used in static hepatocytes in an effort to mimic an *in vitro* environment and to preserve the desired phenotypes. The presence of these hormones in culture media, usually beyond physiologic or even therapeutic concentration,

complicates metabolic studies due to their pleiotropic effects. Our demonstration that many alterations in metabolic phenotypes are actually consequences of hypoxia during static culture warrants reassessment of the necessity of many hormonal additives to culture media.

In conclusion, our study shows that statically cultured hepatocytes are in a state of hypoxia as a result of insufficient oxygen delivery. We demonstrate important metabolic features of hypoxia in statically cultured hepatocytes, including activation of HIF signaling, suppressed fatty acid oxidation, enhanced glycolysis, and excessive lipid accumulation. Shaking the plates during primary hepatocyte culture effectively prevents HIF signaling, corrects altered glucose and lipid metabolism, and mitigates dedifferentiation. Overall, our work suggests that the shaken collagen sandwich culture is an advisable culture method for primary mouse hepatocytes to better maintain differentiation and functions of hepatocytes and to avoid unintended adaptive responses to hypoxia.

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